GR

ORIGINAL SUBMISSION

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Echem Hightech Co.,Ltd. – US Office
331 Bautista Place
San Jose, CA 95126
(650)244-2586

June 8, 2009

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

Subject: GRAS Notice for Lycopene derived from recombinant *E. coli*

Dear Sir/Madam:

According to the proposed rule outlined in 62 Fed. Reg. 18939 (April 17, 1997), Echem Hightech Co.,Ltd. (Echem) hereby submits this notification that the use of the biosynthetic lycopene derived from recombinant *E. coli*, its 10% lycopene oil suspension and its 5% powder products as a nutrient in food is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Echem has determined that such is generally recognized as safe (GRAS) based on scientific procedures.

The information and data relied upon by Echem in making this conclusion is summarized in the enclosed materials. They include:

1. GRAS exemption claim.
2. Conclusions of the expert panel.
3. Biosynthetic lycopene (Basis information for GRAS determination).
4. A published paper on the toxicity findings of biosynthetic lycopene: *Regul. Toxicol. Pharmacol. (2008) vol.52, 163-168.*

To facilitate your review, the format of this submission is based on the proposed 21 C.F.R. § 170.36 (c) (62 Fed. Reg. at 18961); all of the abovementioned information is provided in triplicate.

Sincerely,

(b)(6)

Peter W. Fan, Ph.D.
Consultant of Echem Hightech Co. Ltd.

Enclosures

GRAS EXEMPTION CLAIM

We hereby claim that the use of Echem's lycopene derived from recombinant *Escherichia coli*, including lycopene crystal, 5% powder, and 10% oil suspension products as a source of the nutrient lycopene in foods is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because we have determined that such use is generally recognized as safe (GRAS).

(1) Name and address of the notifier

Ming-Hsi Chiou
Biotech Division Manager
Echem Hightech Co., Ltd.
No. 2, Szu-Wei Road, Hsin-Chu Industrial Park
Hu-Kou Hsieng, Hsin-Chu Hsien 303
Taiwan

(2) Name of GRAS Substance

Biosynthetic lycopene from recombinant *Escherichia coli*, LycoBest (trade name)

(3) Product description

Echem's all-trans lycopene derived from recombinant *Escherichia coli* is a dark-red crystalline powder that is identical to the lycopene that occurs naturally in tomatoes. The crystalline lycopene is used in the manufacture of 5% powder and 10% oil suspension products.

(4) Intended Use and Food Use Levels

Biosynthetic lycopene derived from recombinant *Escherichia coli* is intended to be added to foods as nutrient supplements to increase the dietary intake of lycopene. These foods include baked goods, beverages, breakfast cereals, cheeses, frozen dairy desserts, milk products, snack foods, soft candy, processed fruits and fruit juices which contain very little or no lycopene. The food use levels for lycopene are no more than 50 milligrams of lycopene per kilogram of food (ppm) based on previously published proposal from Vitatene Company in 2005. The intended use is not for the purpose of imparting color although lycopene has the effect of color. Echem will

submit a separate color additive petition for FDA premarket approval should manufacturers express a desire to use Echem's lycopene as a color additive.

(5) Basis for the GRAS determination

Echem's determination of the GRAS status of biosynthetic crystalline lycopene derived from recombinant *Escherichia coli* for use as a nutrient supplement is based on scientific procedures.

(6) Availability of Information

The data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request, or are available for FDA's review and copying at reasonable times at the offices of the notifier.

(b)(6)

Ming-Hsi Chiou
Biotech Division Manager
Echem Hightech Co., Ltd.

Please send all correspondence to:

Peter W. Fan, PhD
Echem Hightech Co., Ltd. – US Office
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San Jose, CA 95126

CONCLUSIONS OF THE EXPERT PANEL:

THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF LYCOPENE FROM RECOMBINANT *ESCHERICHIA COLI*

Prepared for Echem.

Hsinchu, Taiwan

Prepared by

THE EXPERT PANEL

May 12, 2009

Instruction

At the request of Echem Hightech Co., LTD (Echem), an Expert Panel of independent scientists, qualified by relevant experience and scientific training to evaluate the safety of food ingredients, was convened to review the safety and determine the generally recognized as safe (GRAS) status of lycopene that derived from recombinant *Escherichia coli*. The Expert Panel included:

Dr. Wen-Shen Chu ,
Senior Research Scientist & Vice Head, Bioresource Collection and Research Center at Food Industry Research and Development Institute, Hsinchu, Taiwan

Signature: (b)(6) Date: May 26, 2009

Dr. Shi-Yen Shiau
Professor
Department of Food and Nutrition, Providence University, Taichung, Taiwan

Signature: (b)(6) Date: May 15, 2009

Dr. Ning-Sing Shaw
Professor
Department of Biochemical Science and Technology, National Taiwan University, Taiwan

Signature: (b)(6) Date: May 20, 2009

Dr. Ming-Ju Chen
Professor
Department of Animal Science and Technology, National Taiwan University, Taiwan

Signature: (b)(6) Date: May 25, 2009

Dr. Yu-Wen Cheng
Associate Professor
Department of Pharmaceutical Analysis, Taipei Medical University, Taiwan

Signature: (b)(6) Date: May 26, 2009

CONCLUSIONS OF THE EXPERT PANEL: THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF LYCOPENE FROM RECOMBINANT *ESCHERICHIA COLI*

The Expert Panel, independently and critically examined a comprehensive package of publicly available scientific information and data from the literature and the other published sources. In addition, the Expert Panel also evaluated the data and information about biosynthetic lycopene derived from recombinant *E. coli* that provided by Echem. After independent critical evaluation of the available data and information, the collective conclusions of the Expert Panel follow:

- The substance that is subjected to this generally recognized as safe ("GRAS") determination is crystalline lycopene produced by rDNA technology. This material is used in the manufacture of Echem's commercial products, including LycoBest Lycopene Crystal, LycoBest 5% Powder, and LycoBest 10% Oil Suspension, which are intended to be added to foods as nutrient supplements to increase the dietary intake of lycopene.

- The LycoBest Lycopene Crystal is produced by a unique lycopene production method via rDNA technology after fermentation, solvent extraction, recovery, and purification. Strain *Escherichia coli* M_2H, containing foreign genes as the fermentative production strain, is derived from *Escherichia coli* K12. Published evidence has demonstrated that *E. coli* K-12 is non-pathogenic and non-toxigenic. *E. coli* K-12 meets the safety requirements for GILSP (Good Industrial Large Scale Practice) of OECD (Organization of Economic Co-operation and Development) regulation. *E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA (Toxic Substances Control Act).

- *E. coli* K-12 was engineered to produce lycopene by expressing *dxs, idi, crtB, crtI,* and *gps* genes. The *dxs* and *idi* genes, encoding 1-deoxy-D-xylulose-5-phosphate synthase and isopentyl diphosphate isomerase respectively, were cloned from the genomic DNA of *E. coli* K12. The *crtB* and *crtI* genes, encoding phytoene synthase and phytoene desaturase respectively, were cloned from the genomic DNA of *Erwinia uredovora.* The *gps* gene, encoding geranylgeranyl diphosphate synthase, was cloned from the genomic DNA of *Archaeoglobus fulgidus.*

- *E. uredovora* [*Pantoea ananas* pv. *uredovora*], a facultatively anaerobic Gram negative rod bacterium, is a common plant pathogen of fruits and vegetables causing soft rot diseases. It attacks uredia of *Puccinia graminis* and can exist in soil. Extensive literature searches have not identified scientific data implicating *E. uredovora* as a human pathogen. *A. fulgidus*, a sulphur-metabolizing microorganism, belongs to the genus Archaeoglobales. The known Archaeoglobales are strict anaerobes. Most of Archaeoglobales are hyperthermophilic marine sulphate reducers found in hydrothermal environments. They grow between 60 and 95℃ with an optimum at 83℃. The organisms are organoheterotroph, using a variety of carbon and energy sources. They can also grow lithoautotrophically on hydrogen, thiosulphate, and carbon dioxide. The genome of *A. fulgidus* type strain VC-16 has been sequenced. Extensive literature searches have not identified scientific data implicating *A. fulgidus* as a human pathogen.

- Echem's crystalline lycopene produced by rDNA technology is extracted and purified from recombinant *E. coli* host cells. The typical HPLC profile of the crystal showed the predominant form of LycoBest crystal is the *all-trans* lycopene that is identical to the lycopene from chemical synthesis and extracted from tomatoes or fungal biomass (*Blakeslea trispora*). The results of UV assay showed that lycopene content is higher than 96% with an average of 99.5%. Content of all-*E*-lycopene analyzed by HPLC demonstrated that it is higher than 90% and the average content is 93.8%.

- In crystalline lycopene analysis, there is a minor by-product (~3.5%) found during the HPLC analyses of lycopene, which was identified as 1-hydroxylycopene on the basis of UV analysis, IR absorption, and the NMR analysis. 1-Hydroxylycopene, also called lycoxanthin, is the product of the oxidative reaction of the lycopene in the *E. coli* cell. It is a major nature carotenoid in ripe berries of *Solanum dulcamara* and a minor carotenoid in fruits of tomato. It also presents in some fungi and photosynthetic bacteria. The absorption spectrum (λ_{max} in ethanol at 445,470,502 nm) of the lycoxanthin was identical to that of lycopene but with the polarity of a monohydroxycarotenoid. The biological significance of the occurrence of this compound is not known and no adverse effect on the ingestion of lycoxanthin has been reported in the literatures. Literature review indicates that the oxygenated carotenoids compounds may be present in larger quantities in over-ripe tomatoes.

- The purified lycopene crystals from recombinant *E. coli* is then formulated into either a 10% oil suspension or 5% powder. Other ingredients of these two formula, including corn starch, α-tocopherol, vitamin C, corn oil, and gelatin, are also food grade. The specifications of LycoBest Lycopene Crystal, LycoBest 5% Powder, and LycoBest 10% Oil Suspension are set to assure food grade products.

- A rat toxicity study of Echem biosynthetic crystalline lycopene product demonstrated the safety of ingestion of biosynthetic crystalline lycopene. In an acute and 28-day subacute toxicity oral dosing study, no adverse effects were observed at doses of 0, 200, 500, and 2000 mg/kg-bw/day of the product tested (10% biosynthetic lycopene). The no-observed adverse effect level (NOAEL) for this study was concluded to be 2000 mg/kg-bw/day (highest dose tested) for the product tested, which is equivalent to 200 mg biosynthetic lycopene/kg-bw/day based on the lycopene content of 10% in the product tested. The human safety margin from food consumption and dietary supplement intake is calculated to be 20 mg per day, thus providing an approximate 600-fold safety margin. The result is similar to those of toxicity studies on lycopene products derived from tomato oleoresin extract, chemical synthesis, or *B. trispora*, all of which have received FDA GRAS Notification.

- Lycopene as a dietary supplement is considered to be well tolerable, due to the absence of any adverse effect in published human clinical and animal studies to date. The dosage up to 75.0 mg per day of lycopene was tested and no significant adverse biological effect or illness was reported. Carotenodermia appeared in a few people treated with high dosage of lycopene for a long-term period. This symptom gradually reversed when lycopene consumption was stopped.

- Daily intake of lycopene is different due to dietary habits. The estimated daily consumption of lycopene from natural food sources is reported to be approximately 5~10 mg/day in the United States. Several studies indicate benefits of lycopene at a consumption level of approximately 25-30 mg lycopene/day. Lycopene from recombinant *E. coli* is intended for use as a food ingredient and as a dietary source of the nutrient lycopene in foods such as baked goods, beverages, breakfast cereals, cheeses, frozen dairy desserts, milk products, snack foods, soft candy, processed fruits, and fruit juices. In these intended use food, the maximum use-level for lycopene is less than 50 mg/kg. The use-levels for lycopene in these foods follow the Vitaene's estimation in 2005 GRAS Notice for lycopene derived from *Blakeslea trispora*.

Proposed food uses and use-levels for Echem's biosynthetic lycopene in the U.S.	
Food category	Maximum use-levels for lycopene (ppm)
Baked goods and baking mixes	50
Beverages and beverage bases	25
Breakfast cereals	50
Cheeses	5
Frozen dairy desserts and mixes	25
Milk products	50
Processed fruits and fruit juices	25
Snack foods	30
Soft candy	25

The use-levels for lycopene follow those of the Vitatene Company proposed in 2005.

- In conclusion, the safety of biosynthetic lycopene from recombinant *E. coli* and the formulated products is supported by experimental animal studies and analytical specifications and data, which indicate that dietary lycopene does not produce adverse effect on body weight gain, organ weight, clinical chemistry, hematology, urinalysis or gross observation. Furthermore, from literature reviews, the bioavailability of lycopene from processed tomoto products approaches that of purified natural sources or synthetic forms of lycopene. Review of the clinical data and epidemiology studies of lycopene intake show no adverse effect over a wide range of exposures. Therefore, we, the Expert Panel, based on scientific procedures, conclude that biosynthetic lycopene from recombinant *E. coli*, meeting food grade specification, is generally recognized as safe (GRAS) to be used as dietary ingredient in food.

Biosynthetic Lycopene

(Basis Information for GRAS Determination)

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Content

1. Description of LycoBest

[1] We hereby use the term "LycoBest" to denote biosynthetic lycopene from *E. coli* using recombinant DNA technology.

LycoBest is the trade name of Echem's lycopene and produced by the procedures of recombinant DNA technique, fermentation, extraction and purification. The purity of LycoBest™ Lycopene Crystal can reach higher than 96%. LycoBest 5% powder and LycoBest 10% Oil Suspension are the formulated serial products.

Because of different manufacturing procedures, in comparison with other lycopene sources such as that derived from tomato extract, chemical synthesis and fungal fermentation, LycoBest products can achieve purity of 96% or higher. The lycopene crystal, 10% lycopene oil suspension and 5% lycopene powder have passed through rigorous examinations of product specification, stable storage and microorganism safety to guarantee product quality.

Toxicity experiments of LycoBest in animals were carried out by an independent contracting company, Green Seasons Biotech Co., Ltd. Green Seasons' pre-clinical safety evaluations and drug validations are certified by the Chinese National Laboratory Accreditation (CNLA) and are in compliance with other relevant international rules and regulations as well as non-clinical GLP (Good Laboratory Practice) for drugs. Moreover, the company's proposal design, experiment procedures, and report quality are conformable to relevant international standards and major inspection authorities' requirements. The results showed that the SD rats after 28 days of continuous oral administration of experimental material "Lycopene", no observed adverse effect level (NOAEL), was observed up to dosage of 2,000 mg/Kg. The paper was published in ***Regul. Toxical. Pharmacol. (2008) vol. 52, 163-168.***

2. Biosynthetic lycopene from recombinant DNA technology

2.1 Properties of lycopene

2.1.1 Common Name or Usual Name

Lycopene

Echem's lycopene is derived from recombinant *Escherichia coli*. The proposed trade name is LycoBest™.

2.1.2 Chemical name and Chemical Abstract Service (CAS) Number

The predominant occurring lycopene isomer in the final manufactured material is all-*trans* lycopene, the CAS number is [502-65-8]

2.1.3 Empirical formula

Lycopene is a nonpopar hydrocarbon chain with two open-end rings, a molecular weight of 536.87 daltons, and empirical formula $C_{40}H_{56}$ (Merck, 2001).

2.1.4 Structural formula

All-*trans* lycopene is a red crystal powder with melting point of 173℃ that is soluble in fats and certain organic solvents but virtually insoluble in water, methanol and ethanol (Merck, 2001). Various *cis* form isomers of lycopene are shown in figure 1.

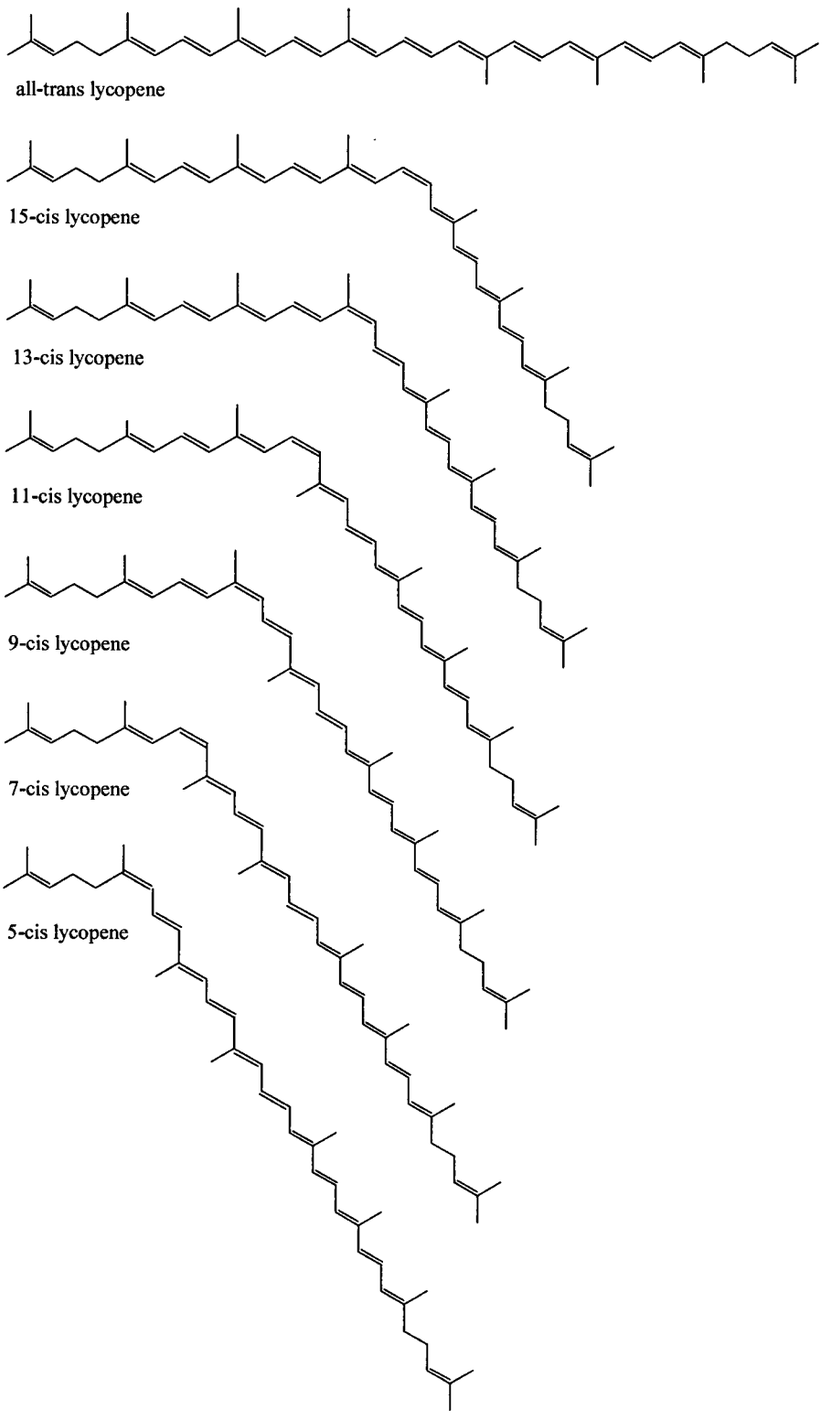
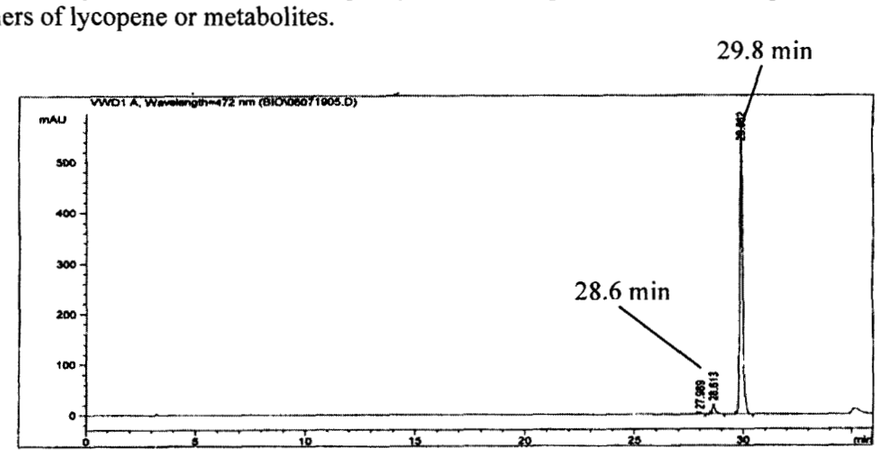


Fig. 1 Isomers of lycopene

2.2 Biosynthetic lycopene from recombinant *E. coli*

Echem's lycopene produced by recombinant DNA technique is extracted and purified from recombinant *E. coli* host cell. The typical HPLC profile of LycoBest crystal is shown in figure 2 and the predominant form of LycoBest crystal is the *all-trans* lycopene form. The peak at 29.8 min elution time corresponded to the form from LycoBest crystal of at least 90% in purity. The other peaks of the HPLC profile are the isomers of lycopene or metabolites.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=472 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	27.989	VV	0.1173	30.74016	3.87673	0.5801
2	28.613	BB	0.1354	188.17010	20.36989	3.5513
3	29.862	BB	0.1345	5079.77588	570.13739	95.8686
Totals :				5298.68615	594.38401	

Fig. 2 Typical HPLC profile of crystalline lycopene from recombinant *E. coli*

2.3 Analysis of by-product from LycoBest crystal

The compound present at retention time near 28.6 min (2.5%~4%) in a typical HPLC profile (Fig. 2) has absorption spectrum identical to lycopene. Mass spectrometer analysis of this peak indicated that it is 16 mass units higher than that of lycopene. Infrared spectroscopy demonstrated the existence a hydroxy functional group. Further 1H-NMR analysis finally confirmed that this compound was 1-hydroxylycopene (lycoxanthin). The byproduct (lycoxanthin) is a known chemical entity [CAS number: 19891-74-8] that is commonly present during production of lycopene from recombinant *E. coli* and is found in nature. It is a major carotenoid in ripe berries of *Solanum dulcamara* and a minor carotenoid in fruits of tomato (Britton et al., 2004). No adverse effect on the ingestion of lycoxanthin has been reported in the literatures.

2.4 Comparison of lycopene from *E. coli* fermentation, *B. trispora* fungal fermentation, chemical synthesis and natural extraction

Production of lycopene can come from different manufacturing procedures. Results comparing these lycopene products from *E. coli* fermentation, *B. trispora* fungal fermentation, chemical synthesis and natural extraction are summarized in table 1 (Vitatene, 2003).

Table 1 Comparison of chemical properties of lycopene from different soures

	Chemical synthesis lycopene	Natural extraction lycopene	*B. trispora* fermentation lycopene	*E.coli* fermentation lycopene (Echem)
purity	≧96%	≧5% (all pigments content)	≧95%	≧96%
Impurity, other pigments	≦0.3% C25 aldehyde	other pigments, oil, lipid, wax and nature spice	other carotenoid	other carotenoid (lycoxanthin)
All-trans isomers	>70%	94-96%	≧90%	≧90%
5-*cis*-isomers	<25%	3-5%	1-5%	<5%
9- *cis*-isomers	<1%	0-1%		
13- *cis*-isomers	<1%	1%		
Other *cis*-isomers	<3%	<1%		
Final product	10% lycopene (5 % ascorbyl palmitate and 1.5% α-tocopherol)	Oleoresin : 2-3% lycopene Lycopene powder: 5% lycopene	5-20% oil suspension with α-tocopherol suspension (1% of lycopene level)	Lycopene Crystal, 10% Oil Suspension and 5% Lycopene powder

3. Product specification, method of analysis, and product description

LycoBest Lycopene Crystal, LycoBest 5% Powder and LycoBest 10% Oil Suspension are products of Echem derived from recombination DNA technique, fermentation, extraction, purification and formulation procedures. The specification and results of lycopene content, heavy metal analysis, Mycotoxin and microbial safety tests are shown in tables 2, 3 and 4.

3.1 Specifications of LycoBest lycopene crystal

3.1.1 Specification

The specifications of LycoBest lycopene crystal majorly followed the criteria of the United States Pharmacopea (USP 29). The content of Lycopene is 96% or higher (≧96%) by UV assay.

3.1.2 Methods of assay

The assays are based on USP-29.

3.1.3 Results of assay

Three batches of LycoBest lycopene crystal products were analyzed. The batch numbers are Ly961219C, Ly961126C and Ly970106C.Results of assay showed that lycopene content is higher than 96% and the average content is 99.5%. Content of all-*E*-lycopene analysis by HPLC demonstrated that its content is higher than 90% and the average content is 93.8%.

Table 2 Specifications of LycoBest Lycopene Crystal

Batch No.Ly961219C

Analysis	Specification	Value
Appearance	Dark Reddish crystal	Confirms
Identification(cyclohexane, λ_{max})	476±1.0 nm	476.0 nm
Identification(UV-Vis, A_{476}/A_{508})	1.12±0.02	1.12
Content of lycopene(UV-Vis)	≧96.0 %	99.5%
Content of all-*E*-lycopene (HPLC)	≧90.0 %	93.7%
Loss on drying	≦0.2%	0.0%
Residue on ignition	≦0.2%	0.1%
Heavy metals	＜10 μg/g	passed
Residual Solvent (Methylene chloride)	≦600 ppm	123 ppm
Residual Solvent (n -Hexane)	≦290 ppm	35 ppm

Batch No.: Ly961126C

Analysis	Specification	Value
Appearance	Dark Reddish crystal	Confirms
Identification(cyclohexane, λ_{max})	476±1.0 nm	476.0 nm
Identification(UV-Vis, A_{476}/A_{508})	1.12±0.02	1.12
Content of lycopene(UV-Vis)	≧96.0 %	98.3%
Content of all-*E*-lycopene (HPLC)	≧90.0 %	93.1%
Loss on drying	≦0.2%	0.0%
Residue on ignition	≦0.2%	0.1%
Heavy metals	＜10 μg/g	passed
Residual Solvent (Methylene chloride)	≦600 ppm	283 ppm
Residual Solvent (n -Hexane)	≦290 ppm	111 ppm

Batch No.: Ly970106C-1

Analysis	Specification	Value
Appearance	Dark Reddish crystal	Confirms
Identification(cyclohexane, λ_{max})	476±1.0 nm	476.2 nm
Identification(UV-Vis, A_{476}/A_{508})	1.12±0.02	1.12
Content of lycopene(UV-Vis)	≧96.0 %	100.9%
Content of all-*E*-lycopene (HPLC)	≧90.0 %	94.8%
Loss on drying	≦0.2%	0.0%
Residue on ignition	≦0.2%	0.1%
Heavy metals	＜10 μg/g	passed
Residual Solvent (Methylene chloride)	≦600 ppm	442 ppm
Residual Solvent (n -Hexane)	≦290 ppm	185 ppm

3.2 Specification of LycoBest 5% powder

3.2.1 Specification

Specification of LycoBest 5% powder is 5% or higher. Other ingredients of this formula include 45-55% arabic gum, 35-45% lactose, 2-3% α-tocopherol and 1-2.5% vitamin C.

3.2.2 Methods of assay

The assays are based on USP-29.

3.2.3 Results of assay

Three batches of LycoBest 5% powder were analyzed. The batch numbers are LTG005060501, LTG005060502 and LTG005060503. Results of assay showed the content of lycopene, heavy metals, aerobic count and solvent residue conform to specification. Data are shown in table 3.

Table 3 Specification of LycoBest 5% powder

Batch No.: LTG005060501

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Powder	Confirm
Content(UV-Vis)	USP 29	≧5.00 %	5.16 %
Loss on drying	USP 29	≦8.00 %	6.78 %
Particle size (Thru. No.20)	Mesh selection	≧99.0 %	99.9 %
Particle size (Thru. No.100)	Mesh selection	≦15.0 %	8.1 %
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu /g	$<10^3$ cfu /g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

Batch No.: LTG005060502

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Powder	Confirm
Content(UV-Vis)	USP 29	≧5.00 %	5.25 %
Loss on drying	USP 29	≦8.00 %	7.15 %
Particle size (Thru. No.20)	Mesh selection	≧99.0 %	99.9 %
Particle size (Thru. No.100)	Mesh selection	≦15.0 %	7.1 %
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu /g	$<10^3$ cfu /g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

Batch No.: LTG005060503

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Powder	Confirm
Content(UV-Vis)	USP 29	≧5.00 %	5.78 %
Loss on drying	USP 29	≦8.00 %	5.85 %
Particle size (Thru. No.20)	Mesh selection	≧99.0 %	99.7 %
Particle size (Thru. No.100)	Mesh selection	≦15.0 %	8.2 %
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu /g	$<10^3$ cfu /g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

3.3 Specification of LycoBest 10% oil suspension

3.3.1 Specification

Specification of LycoBest 10% oil suspension content is 10% or higher. Other ingredients of this formula include 5% α-tocopherol and 85% corn oil.

3.3.2 Methods of assay

The assays are based on USP-29.

3.3.3 Results of assay

Three batches of LycoBest 10% oil suspension products were analyzed. The batch numbers are LF010051201, LF010060401 and LF010060601. Results of assay showed the content of lycopene, heavy metals, aerobic count and residual solvent conform to specification. Data are shown in table 4.

Table 4 Specification of LycoBest 10% oil suspension

Batch No.: LF010051201

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Oil suspension	Confirm
Content(UV-Vis)	USP 29	≧10.00 %	10.17%
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu /g	$<10^3$ cfu/g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

Batch No.: LF010060401

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Oil suspension	Confirm
Content(UV-Vis)	USP29	≧10.00 %	10.62 %
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu /g	$<10^3$ cfu /g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

Batch No.: LF010060601

Analysis	Method	Specification	Value
Appearance	Visual detection	Dark Reddish Oil suspension	Confirm
Content(UV-Vis)	USP29	≧10.00 %	10.36 %
Total aerobic count	MB-CNT-01-01-1	$<10^3$ cfu/g	$<10^3$ cfu/g
Coliforms	MB-CNT-01-01-2	Negative	Negative
Solvent Residue	USP 29	Meet USP SPEC.	Passed
Heavy metals	USP 29	< 10 ppm	Passed

4. Production Process, storage and the stability of lycopene

4.1 Production flow chart

Echem's lycopene crystal is obtained from high-cell density fermentation production and the host cell is recombinant *E. coli*. *E. coli* biomass is extracted, purified and crystallized to acquire highly purified lycopene crystals.

The manufacture process is divided into two parts. The first part is recombinant *E. coli* fermentation (fermentation flow is shown in figure 3). The second part is extraction and purification (purification procedure is shown in figure 4). After the cells are grown to high density in a fermentation vat, they are concentrated, washed, dried, and then extracted with solvent. Solvent extraction is used to directly purify lycopene inside *E. coli* biomass. The cells do not need be lysed or disrupted before extraction. After crystallizing the lycopene by concentrating the filtrated liquid-phase extract and after drying, high purity lycopene crystal is obtained which meets the specification.

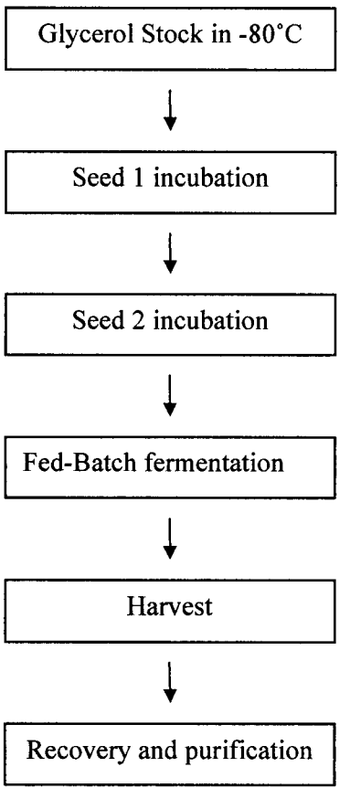


Fig. 3 Flow chart of recombinant strain fermentation

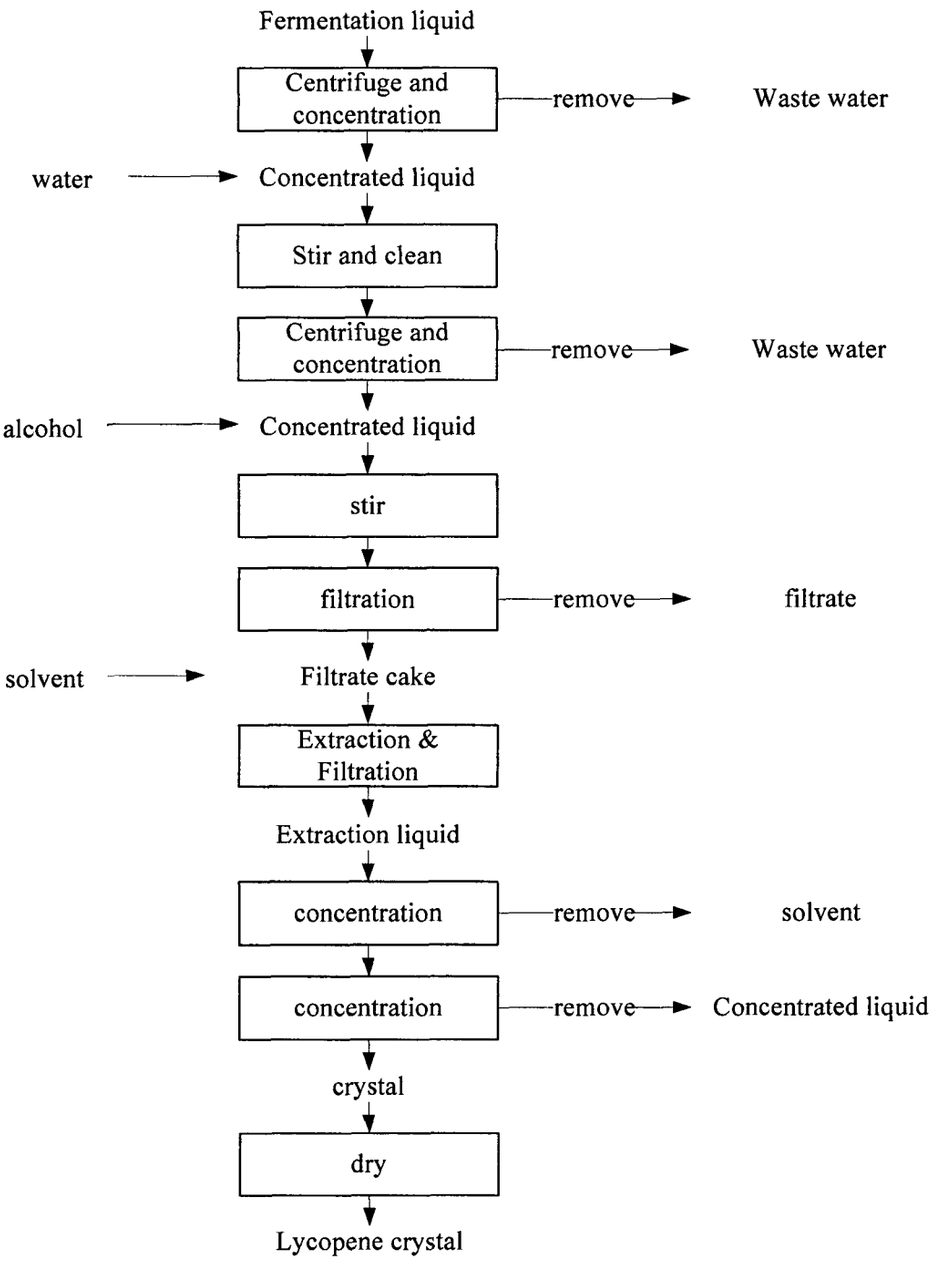


Fig. 4 Flow chart of lycopene recovery and purification

4.2 Formulation process

Highly purified lycopene crystal is added to corn oil containing antioxidative α-tocopherol and constituted to the final product 10% lycopene oil suspension. This product is a thick liquid of dark reddish after a high pressure homogenization and proposed to be used as a food ingredient.

The lycopene crystals are added to a mixture of antioxidant (α-tocopherol & vitamin C), arabic gum and water and mixed evenly. The mixture mentioned above is processed by a high pressure and high speed homogenizer. Lactose is added as a carrier for the product. Low temperature heat blast atomization is followed to makes the granules. This resulting product is 5% formulation powder.

Echem's 5% lycopene powder and 10% lycopene oil suspension are the final products. To avoid oxidative degradation of products, all of the operation conditions are performed under inert gas atmosphere of nitrogen.

4.3 Storage and stability

4.3.1 Storage

Lycopene is a highly conjugated polyene structure and is sensitive to environmental condition factors like temperature, light and oxygen. It is subject to decomposition if stored improperly. Therefore, preparations of LycoBest products are performed in the absence of oxygen to reduce oxidative degradation and decomposition. LycoBest products are best stored at low temperature and filled inert gas such as nitrogen to reduce oxygen contact.

4.3.1.1 LycoBest lycopene crystal

LycoBest lycopene crystal is stored at a temperature below -20°C. After opening the package containing the product, it is recommended that the package be filled with nitrogen and resealed again to reduce oxidative damage.

4.3.1.2 LycoBest 5% powder

LycoBest 5% powder is stored at a dry and low temperature (4°C) environment. Under the recommended storage condition, effective period is 12 months. After opening the package containing the product, it is recommended that the package be filled with nitrogen and resealed again to reduce oxidative damage.

4.3.1.3 LycoBest 10% oil suspension

LycoBest 10% oil suspension is stored at a dry and low temperature (4°C) environment. Under the recommended storage condition, effective period is 12 months. After opening the package containing the product, it is recommended that the package be filled with nitrogen and resealed again to reduce oxidative damage.

4.3.2 Stability of lycopene

Because of the highly conjugated polyene structure, lycopene will degrade or isomerize when it is exposed to light and heat. The stability of lycopene crystal and 10% oil suspension products were studied. Results are summarized in table 5 and table 6. Lycopene crystal is stored at temperature -20°C under nitrogen. Results of their storage stability study are shown in table 5 and figure 5. The result demonstrated that lycopene purity is maintained above 94% up to 6 months of storage. The 10% oil suspension product, lycopene content did not change for three months regardless of storage temperature (4 °C, 25 °C or 40°C).

Table 5 Analysis of product stability of LycoBest lycopene crystal

Examination method	Lycopene content (%)									
	Examination period (month)									
	0	0.5	1	1.5	2	2.5	3	4	5	6
UV (472nm)	102	98	99.4	100.1	98.4	99.7	99.6	97.8	98.1	94.1

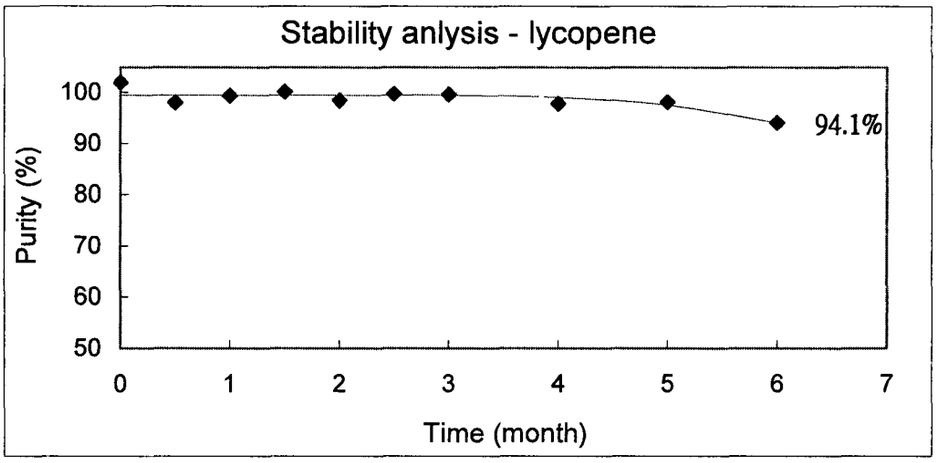


Fig. 5 Analysis of product stability of LycoBest lycopene crystal

Table 6 Analysis of LycoBest 10% lycopene oil suspension product stability

Items	Temperature (℃)					
	4		25		40	
	Time(month)		Time(month)		Time(month)	
	0	3	0	3	0	3
Lycopene content (%)	10.91	10.52	10.91	10.65	10.91	11.07

5 Biosynthesis of lycopene from *E. coli* background information

Lycopene is a secondary metabolite and biosynthesized with related genes including *dxs, gps, crtBI and idi* genes. These genes are from different bacteria: (1) *dxs* & *idi*: control the flux to lycopene from *Escherichia coli*, (2) *gps*: GGPP synthetic gene from *Archaeoglobus fulgidus*, (3) *crtBI*: lycopene synthetic genes from *Erwinia uredovora* (Farmer and Liao, 2001). We engineered an *E. coli* strain to produce lycopene by expressing the necessary biosynthetic enzymes. In order to increase the titer of lycopene, we used traditional UV mutation screening method to isolate a high-lycopene yield mutant, *E. coli* M_2H, as our production strain.

5.1 Lycopene from recombinant DNA technology

Biosynthesis pathway of lycopene from *E. coli* is shown as figure 6.

5.1.1 Origin of biosynthesis gene cluster of lycopene

The *dxs* gene encodes of 1-deoxy-D-xylulose 5-phosphate synthase is cloned from *E. coli* genomic DNA.

The *gps* gene encodes of geranylgeranyl diphosphate synthase is cloned from *Archaeoglobus fulgidus* genomic DNA.

The *crtB* gene encodes of phytoene synthase is cloned from *Erwinia uredovora* genomic DNA.

The *crtI* gene encodes of phytoene desaturase is cloned from *Erwinia uredovora* genomic DNA.

5.1.2 The regulation gene

The regulation gene, *idi* gene, encodes of isopentyl diphosphate isomerase is cloned from *E. coli* genomic DNA which will improve production yield of lycopene.

5.2 Source of bacteria

5.2.1 *Escherichia coli* M_2H

Strain *Escherichia coli* M_2H, contained foreign genes used as fermentation production strain, is derived from *Escherichia coli* K12. *Escherichia coli* K12 is a nonpathogenic host. It meets the safety requirements for GILSP (Good Industrial Large Scale Practice) of OECD (Organization of Economic Co-operation and Development) regulation.

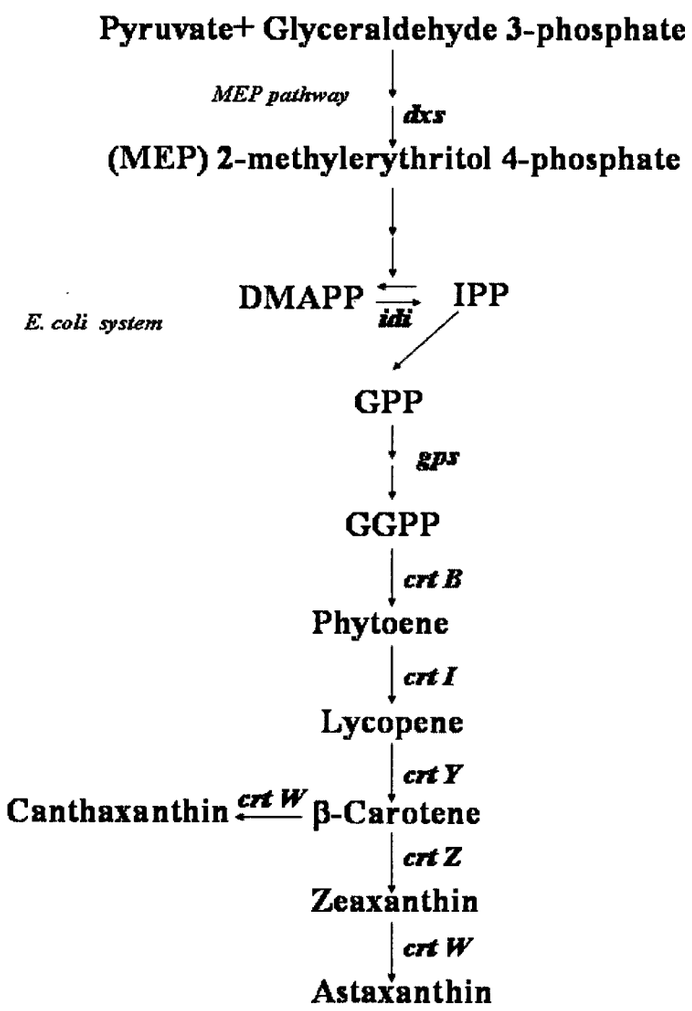


Fig. 6 The biosynthesis pathway of lycopene

6. Global regulations on the uses and daily allowances of lycopene

6.1 Current uses of lycopene worldwide

6.1.1 Natural occurrence of lycopene in the diet

Lycopene is one of the major carotenoids presented in red fruits and vegetables, including tomatoes, watermelon, pink guavas and pink grapefruits, as well as in fungi and algae. According to the different species and ripening extent, the lycopene concentration of tomatoes can range from 3.1 to 7.7 mg/100g, levels are as high as 40 mg/100g tissue (Nguyen and Schwartz, 1999). Depending on dietary intake, lycopene presented to be a predominant carotenoid in human plasma and contributing from 21 to 43% of total serum carotenoids, with similar constituent found in men and women (Vitatene, 2003).

6.1.2. Intake of lycopene in the world

The consumption of dietary lycopene has been studied in variety populations. A British study investigated with elderly females, has reveal that ingestion of high lycopene content food such as tomatoes and tomatoes related products, the daily intake lycopene concentration would reach 1.03 mg per person. Lycopene concentration varieties in plasma also arising from seasonal variation had been found especially in summer and autumn (Scott *et al.*, 1996). From the study report of Olmedilla et al. (1994), lycopene or serum carotenoid levels in a Spanish population had revealed no seasonal variation. The estimated mean daily intake of lycopene from natural food sources is reported to be approximately 8.2mg/day in the United States population (Matulka et al., 2004). For Canadian adults with age 18 to 65 years, mean and median consumptions of lycopene were 6.3 and 1.3 mg per day (Johnson-Down *et al.*, 2002). Using the report from Vitatene pointed that the lycopene intake for general U.S. people estimated to be approximately 4.7 mg per day for chronic mean and 11.3 mg per day for 90^{th} percentile (Vitatene, 2003).

6.1.3 Using of lycopene as a Color Matter and Dietary Supplement

Lycopene extract from tomatoes is authorized as a color and as an available dietary supplement by Europe and The U.S. (E160d, Directive 94/36/EC; US CDR 21 73.295). Synthetic lycopene is considered Generally Recognised as Safe (GRAS) for use as a food ingredient in the U.S. but is not approved for coloring additives (GRAS Notice No. GRN 000119). The lycopene from *B. trispora* has been accepted for use as a coloring matter for foodstuffs by the European Scientific Committee on Food (SCF) (Vitatene, 2003).

6.2 Current status of lycopene from different sources by different manufacturers worldwide

Solvent extracted lycopene from tomatoes is authorized as a coloring agent for food stuffs throughout Europe, the US, and Japan. Synthetic lycopene is currently not approved for coloring matters within the US, E.U or in Japan. In addition, lycopene from

B. trispora has been accepted for use as a coloring agent for foodstuffs in the E.U. In Taiwan, solvent extracted lycopene from tomatoes is authorized as a coloring agent. The scope and application standards of synthetic lycopene also were notified in 2008. Synthetic lycopene can be used as a color or nutrient supplement in food according to the regulation.

A list of current commercial manufacturers as well as the date of application for approval is shown below:

6.2.1 Roche (chemical synthesis)

Roche launched its own synthetic lycopene in different formulations and announced "self-affirmed GRAS" based on independent research studies from different laboratories dated on Sept 6, 2002.

6.2.2 BASF (chemical synthesis)

BASF's 6% formulated lycopene from chemical synthesis received GRAS Notice No. 000119 from FDA on April 7th, 2005.

6.2.3 LycoRed (tomato extract)

LycoRed's 6% formulated lycopene from tomato extract GRAS Notice No. 000156 from FDA on Febrary 7th, 2005.

6.2.4 Vitatene (fermentation)

6.2.4.1 United States

Vitatene's lycopene in different formulations from *B. trispora* fermentation received GRAS Notice No. 000173 from FDA on June 22nd, 2005.

6.2.4.2 Europe

Vitatene applied to European parliament and of the council for approval of lycopene from *B. trispora* fermentation as novel food and novel food ingredients (Regulation (EC) No. 258/97) and was approved on April 6th, 2004.

7. Nutritional information

7.1 Breakdown of formulation ingredients used in LycoBest

Production flow of lycopene from *E. coli* is divided into two parts. First part is fermentation, utilizing large scale bacterial fermentation to produce lycopene in sufficient quantity. Second part is extraction and purification, using solvent to remove lycopene from *E. coli*, producing high purity crystal lycopene. After formulation, lycopene in oil suspension or powder form as end-product will provide customers with ease of use and flexibility to manipulate. Process involved in extraction, formulation, and packaging is performed under nitrogen to minimize loss of lycopene due to oxidation.

7.1.1 Corn oil

Food grade corn oil is used in the formulation of product in oil suspension.

7.1.2 α-tocopherol and vitamin C

Food grade α-tocopherol and vitamin C are used in the formulation of products in oil suspension and powder form as an antioxidant.

7.1.3 Arabic gum and lactose

Food grade arabic gum and lactose are used in the formulation of 5% powder.

7.2 Absorption of lycopene in humans

Lycopene is a fat-soluble chemical substance. Its absorption in humans by small intestine is similar to that of fat absorption after regular meal consumption. Lycopene is absorbed in the intestine after its release from food particles, with the aid of soluble fats and bile acids. Factors that influence the release of lycopene include: The amount and the form of lycopene suspended in food particles; food particle size after chewing and ingestion; and the rate of digestion in the stomach, these factors, together with different cooking methods, will influence the amount of lycopene released. It is known that bioavailability of lycopene is increased after heating carotenoid (including lycopene) rich vegetables. Regarding factors that influence fat absorption, insufficient bile acid secretion or improper fat absorption due to illness will all influence the absorption of lycopene in the intestine (Lu, 2001).

Rao et al. (2004) performed a long term study on the effect of consumption of large quantities of tomato products in 17 healthy volunteers. Bioavailability of lycopene was analyzed. Two weeks prior to the study, subjects were not allowed to consume any tomato-based products in order to obtain a baseline for lycopene. Upon initiation of study, tomato-based products such as tomato juice, sauce, paste, and spaghetti sauce were consumed at approximately 30 mg of lycopene/day for four weeks. Analysis of the serum showed lycopene concentration was significantly increased ($P<0.05$) from 181.79 ± 31.25

nmol/L to 684 ± 113.91 nmol/L. The results of the study suggest that consumption of large quantity of tomato-based products can effectively increase serum concentration of lycopene.

Results from a study conducted by Wu et al. (2003) indicated that lycopene found in food is primarily the more stable all-trans form. However, in blood plasma from human or rodents, approximately 50 to 70% of lycopene is in the cis-form. Among the 114 human volunteers in a 3- to 4-year long term study conduced by Wu, in which the lycopene serum concentration and the cis-trans isomeric ratio in vivo were measured and compared, results showed that there was no significant inter-individual variability, regardless of source of food and effect of metabolism.

7.3 Bioavailability of lycopene

Absorption of carotenoids (including lycopene) can be divided into four steps: 1) Breakdown and digestion of food particles; 2) micelle formation after mixing carotenoids with fat particles; 3) uptake and absorption of carotenoids by mucosal intestinal cells; and 4) with the aid of lymphatic system, carotenoids are distributed throughout blood circulation. Fat found in regular meals is an important player in the absorption of carotenoids in the abovementioned steps. In order to effectively absorb carotenoids, after intake of food, fat found in meals will stimulate bile production, which in turn aids carotenoids to form fat-soluble micelles together with phospholipids and triglycerides (also found in meals). These fat soluble micro particles are then taken up by the mucosal intestinal cells (Lu, 2001).

Carotenoids found in vegetables are either crystal or complexed with proteins. Upon mild heating, the protein-carotenoid complex is destroyed aiding the absorption of carotenoids. The heating process will also aid the solubilization of crystal carotenoids in fat. For instance, serum concentration of lycopene is 2.5 times higher in volunteers who consumed tomato paste than those who consumed fresh tomatoes (Lu, 2001).

A study report based on Dong (2002) pointed out that the bioavailability or the level of absorption of lycopene is influenced by several factors including 1) the number of cis/trans bonds in the molecule; 2) the content of lycopene in food particles; 3) food particle size; 4) the degree of its complexation with proteins, fibers, and fats in food; and the form of lycopene (crystal vs. protein-complex) in vegetables. Lycopene is the major and the most concentrated caroteinoids found in human serum with half life of 2 to 3 days in human. Generally speaking, all-trans lycopene has a better stability than the cis-form. However, because the cis-form forms fat soluble micelles with bile acids more readily than that of all-trans form, it is absorbed more efficiently by human. Therefore, the bioavailability of the cis-form is higher than that of all-trans form. In addition, the components of food particles will affect bioavailability of lycopene as well.

Gartner *et al* (1997) compared the concentration and the bioavailability of carotenoids found in the fat soluble micro particles in a single dose study (approximately 23 mg) between fresh tomato and tomato paste (both formulated with 15 g of corn oil).

The study showed that the cis-trans isomeric ratio were the same between fresh tomato and tomato paste. The level of triglyceride found in the micro particles did not change significantly. Among the volunteers (three males, two females; ages 32 ± 7.3; and BMI (Kg/m^2) of 23.3 ± 4.0), serum concentration of lycopene is 2.5 times higher in subjects who consumed tomato paste versus those who consumed fresh tomato ($P<0.05$ and $P<0.005$, respectively; statistically significant). The level of cis-forms found in paste and fresh tomatoes are similar. The level of α- and β-carotenes found in them is similar as well. Therefore, the results of Gartner *et al.*'s study in human suggest that tomato paste and processed tomato juice, because of heating and breakdown of tomato plant cell wall and fibers during food processing, will allow more efficient absorption of lycopene. Ultimately the bioavailability of processed tomato products is superior to that of fresh tomatoes. In addition, together with oily meals, lycopene serum concentration is two to three times higher after one day of consumption. Diets rich in fibers may decrease the bioavailability of lycopene. Furthermore, absorption of lycopene is more efficient at low rather than high dose. And taken together with β-carotene, absorption is superior to taking lycopene along.

7.4 Maximum tolerance of lycopene intake

The tolerance of lycopene consumption was assessed in prostate cancer patients and healthy individuals. The dosage up to 75.0mg per day of lycopene was generally well tolerated and no results of any significant disadvantage biological effects or illnesses were reports (Carughi and Hooper, 1994; Agarwal and Rao, 1998; Wright *et al.*, 1999; Chopra *et al.*, 2000; Watzl *et al.*, 2000; Chen *et al.*, 2001; Olmedilla *et al.*, 2002; Chang, S., *et al.* 2005). The effects of dietary carotenoids on skin pigmentation, Carotenodermia, had been revealed in a double-blind study conducted by Postaire *et al.* (1997) occurred in 25% of the healthy volunteers were supplemented with 15mg lycopene per day for 16 weeks. From another report conducted by Olmedilla et al. (2002) had not found any objective symptoms of skin discoloration. In many clinical data reveal that lycopene as a dietary supplement is considered well tolerable. Carotenodermia has appeared in a few people treated with high dosage of lycopene for a long-term period. Such effects are supposed to be harmless and readily reversible when lycopene consumption is stopped.

7.5 Recommended daily intake and use for Echem's biosynthetic lycopene

Several recent studies have shown that daily intake of 5 to 10 mg of lycopene per day may prevent several chronic diseases including cancers and cardiovascular diseases. A higher dose (25 to 30 mg per day) is required for the prevention of prostate cancer (Rao and Heber, 2002). Therefore, lycopene from rDNA technology is intended for use, as a dietary source of the nutrient lycopene, in foods such as baked goods, beverages, breakfast cereals, cheeses, frozen dairy desserts, milk products, snack foods, soft candy, processed fruits and fruit juices which contain very little or no lycopene. The intended use is not for the purpose of imparting color although lycopene has the effect of color. The food use levels for lycopene from recombinant *E.coli* are listed below:

Food Use Levels for Lycopene from rDNA technology	
Food Category	Maximum Use-Levels (ppm)
Baked goods and baking mixes	50
Beverages and beverage bases	25
Breakfast cereals	50
Cheeses	5
Frozen dairy desserts and mixes	25
Milk products	50
Processed fruits and fruit juices	25
Snack Foods	30
Soft Candy	25

The use-levels for lycopene follow those of Vitatene Company proposed in 2005

8. Toxicity and safety test in animals

Lycopene from Echem's fermentation and extraction process has been tested for toxicity in both male and female Sprague Dawley (SD) rats. The toxicity studies were conducted by a nationally recognized laboratory, Four Seasons Biotech and were conducted under the guidance listed in Good Laboratory Practice for Nonclinical Laboratory Study.

It is well documented that SD rats are the species of choice for toxicity studies. They have been chose routinely to carry out different toxicity studies including acute and subacute dosing. They are a well studied model for this purpose. In brief, extensive literature searches were conducted, and the relevant data (e.g., studies reporting post-prandial serum and organ levels of lycopene in rats and humans) were compiled in order to compare the uptake and tissue distribution of lycopene between humans and rats. Based on these data, it was concluded that the rat can be regarded as a useful and appropriate animal model for the study of lycopene. Rats chosen for this study are typically six weeks in age.

During the toxicity study, amount of food intake, body mass, exercise pattern, and clinical signs of animals' health were noted. Other clinical signs monitored included urine inspection, blood and plasma bio-analysis, and ophthalmologic inspections. Before the start of the experiment, health conditions were noted and samples (blood and urine) were taken from pre-dosed animals for inspection/analysis. Clinical signs of the animals were noted twice per day (including weekends and holidays) for the duration of the toxicity experiment. Abnormal symptoms or unexpected deaths of animals were also recorded. Date of deaths as well as the time of abnormal symptom development was carefully noted in the record book. Autopsies were performed on dead animals and the possible cause of death was noted.

8.1 Single dose acute oral toxicity test

The present study was to investigate potential acute oral toxicity in rats by a single dose of "Lycopene" at 5 g/kg. A total of 12 rats in each sex were divided into control (corn oil), and treatment group (Lycopene, 5 g/kg). Following 14-day observation, all animals were sacrificed and necropsied.

1. Clinical observations: During the study, no clinical sign was observed in rats of either control or treatment group.
2. Mortality: There was no death observed in rats of control and treatment groups.
3. Variation of body weight: The body weight gain of rats in treatment group was of no significant difference from that of the control group.
4. Food consumption: No significant difference was showed between the treatment and control groups.
5. Gross observations: No gross lesion was observed in control and treatment groups.

In conclusion, based on the afore-said, the rats received a single oral dose of "Lycopene" at 5 g/kg did not show toxicity.

8.2 28-day subacute oral toxicity test
(see Regul. Toxicol. Pharmacol. (2008) vol.52, 163-168)

The study was conducted by an independent contractor to investigate the potential subacute toxicity of LycoBest 10% powder in SD rats. A total of 80 male and female rats were divided into four groups, three groups received test article at doses of 200 (low dose), 500 (intermediate dose) or 2000 mg/kg (high dose) and one control group of vehicle sterile water for injection. Each group consisted of 10 males and 10 females. All animals were sacrificed after 28-day oral administration. The results were summarized as follows:

1. Clinical sign: No clinical sign was observed in any dose and control groups.
2. Mortality: No death was observed in any dose and control groups.
3. Body weight: No significantly body weight gain was observed in any dose and control groups.
4. Food consumption: Significant increase in food consumption was noted in the 200 mg/kg dose males at week 1 and 2. Significant decrease in food consumption was noted in the 500 mg/kg dose males at week 2. Otherwise, significant reduction in food consumption was noted in the 2000 mg/kg dose females at week 4.
5. Gross observation: No abnormality was found related to administration of test article.
6. Clinical pathology: Significant decrease in urine volume was noted in 500 and 2000 mg/kg dose males; however it showed significant increase in the 500 mg/kg dose females. Although there were several significant data in the hematology, blood coagulation and clinical chemistry analysis, these values were still in the clinical acceptable range.
7. Organ weight: No significant change was observed related to administration of test article.
8. Histopathology: No lesion was found related to administration of test article. In conclusion, based on the afore-said in the SD rats with 28-day oral administration of "LycoBest", the NOAEL (No-Observed-Adverse-Effect Level) of "LycoBest" in this study was assumed to be 2000 mg/kg in male and female SD rats.

8.3 Literature reference to other toxicity studies in other animals

8.3.1 Natural tomato oleoresin extract

Natural tomato oleoresin extract (NTOE) contains 6% lycopene produced from tomatoes. NTOE was evaluated for toxicological effects, and found the 50% lethal dose (LD_{50}), derived from the acute oral toxicity study, was greater than 5000 mg/kg body weight. The no-observed-adverse-effect level (NOAEL) derived from the 13-week study was 4500 mg/kg/day. Acute dermal toxicity study of NTOE found no toxicity at 2000 mg/kg body weight (LycoRed, 2004). Furthermore, in an acute oral LD_{50} toxicity study

for rats conducted by LycoRed, its product Lyco-O-Mato (tomato oleoresin) containing 5% lycopene was found to be > 5000 mg/kg body weight.

8.3.2 Synthetic lycopene

BASF described results from a published 13-week oral toxicity study conducted in rats fed BASF's commercial synthetic lycopene products (GRAS notice no. GRN000119). BASF concluded that results of the study support a no-observed-adverse-effect-level (NOAEL) for synthetic lycopene of 324 milligrams per kilogram body weight per day (mg/kg bw/day). BASF noted that this amount is approximately 4000-fold higher on a body weight basis than the mean estimated dietary intake (EDI) of synthetic lycopene. BASF also stated that no adverse effects were reported in an unpublished developmental toxicity study conducted in rats and rabbits fed BASF's commercial synthetic lycopene products, and that no mutagenic effects were observed in unpublished genotoxicity studies conducted with BASF's commercial synthetic lycopene products (GRAS notice no. GRN000119).

8.3.3 Lycopene extracted from *Blakeslea trispora* fermentation

Vitatene (2003) showed that a 90-day oral toxicity study of lycopene 5 and 20% oil suspension in experimental animals conducted with male and female Wistar rats was accorded with the OECD guielines. From the toxicity study of Vitatene's LICONAT, lycopene extracted from *Blakeslea trispora* fermentation revealed that no treatment-related effects on neurobehavioral testing and ophthalmologic examinations, and had no differences in average body weight, organ weights or food consumption, or in items of urinalysis, hematology and clinical chemistry between treated and control groups. The gross necropsy did not show any harmful effects in any organ system and had no lycopene-related lesions as demonstrated by histopathological examinations. In summary of these studies results, dosage of intake lycopene up to 1% are well tolerated by male and female Wistar rats and have no effects of toxicity. The no-observed effect level (NOEL) of lycopene was considered to be 1.0% in the diet.

Following the requirement of ICH, a bacteria mutation test and in vitro chromosome aberration test were applied to evaluate the lycopene 20% CWD from *B. trispora* for the genotoxicity safety. The results of these two studies shown that lycopene from *B. trispora* had no evidence of genotoxic activity (Vitatene, 2003).

8.3.3.1 Margin of safety conducted by Vitatene

From the rat sub-chronic study conducted by Vitatene, based on the average NOEL of 601 mg lycopene per kg body weight per day, the human safety margin from food consumption and dietary supplement intake is calculated to be 20 mg per day and provides an approximate 2,000-fold safety margin.

8.3.4 Summary of other lycopene toxicity studies from literature

Results of *in vitro* genetic toxicity with bacterial system were reported consistently negative. Recommended *Salmonella Typhimurium* strains were treated up to 100 μg per plate of lycopene extracted from fruits and vegetables (Rauscher *et al.*, 1998) or water soluble lycopene beadlet (with and without metabolic activation)(McClain and Bausch, 2003), there were negative results reported. Lycopene 10% WS beadlet formulation was applied at doses up to cytotoxic concentrations (not specified), the mutagenicity in mouse lymphoma cells was negative result (McClain and Bausch, 2003).

Mice and human were used as *in vivo* assay systems for lycopene genetic toxicity. Lycopene did not induced chromosomal aberrations in mouse bone marrow cells (Rauscher *et al.*, 1998) or human lymphocytes cells (Pool-Zobel *et al.*, 1997; McClain and Bausch, 2003).

8.3.5 Carcinogenicity studies

Currently there are no reports that suggest lycopene may cause cancer in animals. Instead, reports regarding the potential chemopreventive effects of lycopene have surfaced. Tumor inducer such as Diethylnitrosamine, N-methyl-N-nitrosurea, and 1, 2-dimethylhydrazine (DMD), *N*-methylnitrosourea or 7,12-dimethylbenz[a]anthracene, and Azoxymethane were applied by intraperitional injections or subcutaneous injection into rats at concentration of 5 or 10 mg per kg for 2 weeks to 100 days, there were no adverse effects of lycopene reported (Kim *et al.*,1997; Wang *et al.*, 1989; Narisawa *et al.*, 1996; Sharoni *et al.*, 1997; Jain *et al.*,1999).

8.3.6 Reproductive Toxicity

From reproductive toxicity study conducted by Vitatene, male and female rats were fed 10 to 20 mg lycopene per kg body weight per day in the diet for nearly 200 days before pregnancy. Results from this study, there were no significant effects on fertility, pregnancy, or fetal aberration (Vitatene, 2003).

The synthetic lycopene formulations such as Lycopene 10 Cold Water Dispersion (CWD) and LycoVit® 10% were investigated in female rats and rabbits to evaluate the potential developmental toxicity. Oral exposure to up to 3000 mg per kg body weight per day of Lycopene 10 CWD or LycoVit® 10% in female rats or 2000 mg per kg body weight per day of Lycopene 10 CWD or LycoVit® 10% in rabbits, there were no substance-related evidence of development toxicity.

8.3.7 Safety and clinical studies in humans

Lycopene has been recognized as a safety phytochemical and has specific physiological activity (ILSI, 1999). There is no data to indicate significant adverse effects and the overall safety is supported by a numerous clinical trials. A review of clinical studies from the scientific literature was summarized as table 10. Healthy male and female volunteers were supplemented with lycopene at levels ranging from 0.5 mg per day for 4 weeks to 75.0 mg per day for 1 week. It revealed no related abnormalities from the biochemical and anthropometric measurements including body weights, full blood counts, liver function tests and immune function (Carughi and Hooper, 1994; Agarwal

and Rao, 1998; Wright *et al.*, 1999; Chopra *et al.*, 2000; Watzl *et al.*, 2000; Chen *et al.*, 2001; Olmedilla *et al.*, 2002; Chang, S., *et al.* 2005). Serum lipid profiles from these investigated such as triglycerides and cholesterol levels including total, high density lipoprotein-, or low density lipoprotein were not changed by the lycopene supplement.

For healthy individuals and prostate cancer patients, daily doses up to 75.0mg lycopene were regard has well tolerance, there were no related adverse biological effects been reported. Non-specific gastrointestinal intolerances have been reported in prostate cancer patients supplemented with 30 mg lycopene per day for period of 3 weeks but the effects were considered minor, and were not reported in other separate study conducted with a similar protocol. A report conducted by Vitatene, incidences of carotenodermia had occurred in approximately 25% of the subjects based on supplemented with 15 mg per day of lycopene for 16 weeks, compared with 40% and 95% of the subjects supplemented with lutein and carotene. However, review the clinical data suggested that lycopene used as dietary supplement has well tolerance, carotenodermia occurred in a small percentage of treated population are considered to be harmless and readily convertible once the carotenoid intake is stopped.

8.4 Summary of LycoBest lycopene

The unique lycopene production method via recombinant DNA technique developed by Echem, after fermentation, solvent extraction, recovery, and purification, a highly purified lycopene crystal, LycoBest is produced. Safety evaluation of LycoBest is summarized below:

1. Extensive quality assurance tests, mainly the specifications, lycopene content, heavy metal analysis, Mycotoxin and microbial safety, and storage conditions have been defined and examined for each batch of lycopene produced.
2. Physicochemical properties of LycoBest based on NMR, IR, mass spectrometer, and HPLC are identical to the chemical entity identified as Chemical Abstracts Service Registry Number 502-65-8, "synthetic lycopene", and "natural lycopene".
3. Purity of LycoBest crystal is 96% or greater. It conforms the specifications of synthetic lycopene and lycopene from *Blakeslea trisporia.*
4. After extensive literature searches, the use of lycopene is beneficial to health and currently there are no reported side effects observed in humans after long term use (synthetic, natural or from fermentation).
5. LycoBest crystal tested in the Bradford assay showed no protein residuals left in the product.
6. Strain *Escherichia coli* M_2H, contained foreign genes used as fermentation production strain, is derived from *Escherichia coli* K12. *Escherichia coli* K12 is a nonpathogenic host. It meets the safety requirements for GILSP (Good Industrial Large Scale Practice) of OECD (Organization of Economic Co-operation and Development) regulation.
7. Acute and 28-day subacute toxicity study showed that the SD rats after 28 days of continuous oral administration of experimental material "Lycopene", no observed adverse effect (NOAEL), was observed up to dosage of 2,000 mg/Kg.

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Pages 000048 - 000054 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

SUBMISSION END

AM

Carlson, Susan

From: Peter Fan [peterwfan@yahoo.com]
Sent: Tuesday, September 22, 2009 12:03 PM
To: Carlson, Susan
Subject: RE: GRN 299 reviewers' comments

Attachments: GRAS 299 Withdraw Letter.pdf

GRAS 299
ithdraw Letter.pdf (

Susan,

Please see attached pdf file for the official request for withdraw letter. Thank you.

Best Regards,

Peter Fan

--- On Thu, 9/17/09, Carlson, Susan <Susan.Carlson@fda.hhs.gov> wrote:

> From: Carlson, Susan <Susan.Carlson@fda.hhs.gov>
> Subject: RE: GRN 299 reviewers' comments
> To: "Peter Fan" <peterwfan@yahoo.com>
> Date: Thursday, September 17, 2009, 11:42 AM
> Thank you very much.
> Please also send us a signed hard copy of this request for
> the records.

> -----Original Message-----
> From: Peter Fan [mailto:peterwfan@yahoo.com]
>
> Sent: Thursday, September 17, 2009 12:35 PM
> To: Carlson, Susan
> Cc: minghsi@echemco.com.tw
> Subject: Re: GRN 299 reviewers' comments
>
> Dear Dr. Carlson,
>
> At this point on behalf of EChem Hightech we would like to
> thank
> everyone in your team for their precious time and energy to
> review our
> submission. We are very grateful for your insightful
> comments,
> suggestions and will do our best to compile all the
> necessary data that
> we already have so that we may reformat our submission at a
> later date.
> Once assembled we will schedule a time to meet with you and
> your team to
> discuss proper directions to move forward. At this
> point December of
> this year might be the best. We will keep in touch
> with you to confirm
> a date once we have re-organized our package and have
> assembled our own
> questions as well as responses to your questions.

> In compliance with good business practice we would like to
> ask you (FDA)

000062

> to cease evaluating GRAS application No. 299, Lycopene from
> Escherichia
> coli expressing lycopene biosynthetic enzymes.
>
> We look forward to working with you closely.

> Sincerely,
>
> Peter Fan
> EChem Hightech Consultant
> 650-244-2586 (day)
> 408-971-2649 (Evening)
> 331 Bautista Place
> San Jose, CA 95126
>
>
> --- On Thu, 9/17/09, Carlson, Susan <Susan.Carlson@fda.hhs.gov>
> wrote:
>
> > From: Carlson, Susan <Susan.Carlson@fda.hhs.gov>
> > Subject: GRN 299 reviewers' comments
> > To: peterwfan@yahoo.com
> > Date: Thursday, September 17, 2009, 10:02 AM
> >
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> > GRN 299 reviewers' comments
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> >
> > Dear Dr. Fan,
> >
> >
> > Thank you for participating
> > in the teleconference with the FDA review team this
> past
> > Tuesday. We look forward to working with you in the
> near
> > future.
> >
> > I am attaching the comments
> > from the reviewers with regard to your company's
> current
> > submission, GRN 299. We hope that you find these
> helpful in
> > preparing a new submission. As we mentioned in the
> > teleconference, we would welcome the opportunity to
> meet
> > with you and your team to discuss further, especially
> before
> > you embark on any additional studies.
> >
> > Please understand that the
> > reviewers' comments should not be construed as
> absolute
> > requirements. As you are aware, the GRAS Notification
> > program is a voluntary program and the decision that
> the use
> > of an ingredient is GRAS ultimately rests with the
> notifier.
> >
> >
> > When you are ready to set up
> > a meeting, just send me an e-mail along with some

> proposed
> > dates. My e-mail address is: Susan.Carlson@fda.hhs.gov,
> my
> > phone number is: 301-436-1253.
> >
> Best regards,
> >
> >
> > Susan
> >
> >
> >
> >
> > Susan Carlson, Ph.D.
> >
> >
> > General Health Scientist
> >
> >
> > U.S. Food and Drug
> > Administration
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> >
> > Center for Food Safety and
> > Applied Nutrition
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> > Office of Food Additive
> > Safety
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> > Division of Biotechnology and
> > GRAS Notice Review
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Echem Hightech Co. Ltd US Office
331 Bautista Place
San Jose, CA 95126
(650)244-2586

September 18, 2009

Susan Carlson, Ph.D.
General Health Scientist
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review

Dear Susan,

In compliance with good business practice, EChem Hightech Co., Ltd would like to ask FDA to cease evaluating GRAS application No 299, Lycopene from *Escherichia coli* expressing lycopene biosynthetic enzymes.

Thank you for your attention

Sincerely,

(b)(6)

Ming-Hsi Chiou
Biotech Division Manager
Echem Hightech Co., Ltd.
No. 2, Szu-Wei Road, Hsin-Chu Industrial Park
Hu-Kou Hsieng, Hsin-Chu Hsien 303
Taiwan

(b)(6)

Peter W. Fan,
Consultant for EChem Hightech

Reference List for Industry Submission, GRN 000299

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000048 - 000054	Jian, Wen-Chi; Chiou, Ming-Hsi; Chen, Yung-Tin; Lin, Chi-Nan, Wu, Mei-Chiao; Du, Chi-Jen Du; Chen-Pan, Cleo	Twenty-eight-day oral toxicity study of lycopene from recombinant Escherichia coli in rats	November 2008	Regulatory Toxicology and Pharmacology	Volume 52, Number 2, pgs 163 - 168

NA- Not applicable